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14. ABSTRACT Progression to metastatic castrate resistant prostate cancer (mCRPC) is a significant clinical problem. Although new hormones therapies have shown efficacy they have been largely ineffective in treating mCRPC. It is becoming increasingly clear the immune system plays a key role in long-term outcomes of cancer. Some emerging immunotherapies have been highly effective at treating late stage cancers. However, it is crucial that the immune system recognize cancer-specific or cancer-associated targets. One such target is mutations in tumors that give rise to so-called neoantigens. Some neoantigens can be shared across patients while others are unique. Despite large genomic sequencing efforts in prostate cancer, a comprehensive analysis of immunological targets has been lacking. Here we describe a first-in-principle approach to uncover the immunogenic potential of a specific type of mutation called gene rearrangements. These mutations occur when two unrelated genes fused together, creating a new gene product that may have distinct neoplastic functions. Using integrated transcriptome analysis, we have identified gene fusions with high-confidence predicted MHC class I restricted epitopes in 6 out of 50 patient tumors. One recurrent gene fusion encoded by the TMPRSS2:ERG type VI fusion was detected in 3 patients and contained a predicted HLA*02:01 restricted epitope. In vitro, we identified T cells from healthy donor peripheral blood that recognizes peptides specific for this mutation. Collectively, our data show that prostate tumors harbor targetable neoantigens encoded by gene rearrangements at a low frequency.					
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BRIEF INTRODUCTION & RATIONALE

The progression of high-risk prostate cancer to metastatic castration resistant prostate cancer (mCRPC) is a significant problem where there are no curative therapies currently available to treat this late stage disease¹. Recent clinical trials have suggested that immunotherapy may be a promising approach for prostate cancer. The number one challenge with immunotherapy is the lack of specific antigens that can generate highly potent anti-tumor immune responses. Recent genomic studies across several different tumor sites provide compelling evidence that the cancer mutanome could provide a source of neo-antigens to stimulate T cell responses². Whether prostate tumors harbor mutations that can serve as T cell targets has not been systematically evaluated. For the purposes of this study, we have chosen to focus on identifying mutations that result from gene rearrangements given their high frequency relative to somatic point mutations. Gene rearrangements can yield novel chimeric amino acid sequences (CASQ) at the junction point of the fused genes. The identification of T cells capable of recognizing CASQ may provide a new family of neoantigens that could be exploited for T cell-based immunotherapy. Thus, the goal of this study was three-fold:

1. To establish a tissue collection platform to acquire matched fresh surgical tumor specimens and T cells for immunological studies
2. To develop a robust high-throughput integrated analytical tool to identification high-confidence MHC class I predicted epitopes from CASQ, and
3. To examine the frequency of T cells capable of recognizing CASQ.

RESULTS

Computational Pipeline

As shown in Fig. 1A, we designed a strategy using three existing tools to identify high-confidence MHC class I epitopes from RNA-seq data. As an initial test of this workflow, RNA transcriptome sequencing was carried out on a high grade human prostate adenocarcinoma, LTL-331. DeFuse analysis revealed 63 tumor specific gene rearrangements (Fig. 1B) and of these 3 fusions were found to be between coding exon boundaries (Fig. 1C). These aberrations include DTNB:LIMS1, TMPRSS2:ERG, and PSMG4:PHACTR1. The MHC class I haplotype of LTL-331 was generated from HLAMiner and epitopes spanning these fusions were predicted by IEDB; however none of the three CASQ from LTL-331 were found to contain any HLA predicted epitopes.

Validation in intermediate and high-risk patients from TCGA

To confirm the validity of our pipeline, we analyzed RNA-seq data from intermediate and high-risk prostate adenocarcinomas catalogued in The Cancer Genome Atlas database. This dataset revealed the presence of an average of 16 tumor specific fusion events per patient with a range of 1-69 (Fig. 2A, B). Thirty patients had at least 10 or more fusions while a smaller proportion (n=20) had less than ten fusion events found in their tumor. Of these 30 patients, 28 had tumors carrying a minimum of one chimeric transcript containing a coding region breakpoint (range of 1-13). For this class of fusions, translation and proteome alignment were performed to identify CASQ and their resultant MHC class I predicted epitopes. Despite the prevalence of CASQ, only 6 of these 23 patients were found to have high-affinity (IEDB score <50 nM) MHC class I predicted epitopes.

Recurrent fusions

In a comparative analysis across the patient cohort, we found that 26% shared some number of transcripts encoding CASQ. Fifty-one different CASQ presented within single tumours as unique fusions, while 4 recurrent fusions were identified within this population (Fig. 3A). Two patients presented fusions between GPBP1 and MTRR as well as two patients with KDM2B and CAMKK2 fusions, nine patients carried TMPRSS2:ERG fusions, and eleven tumours contained a genomic fusion between KLK2 and KLK3 (Fig. 3A). Fusions between TMPRSS2 and ERG have been well annotated, thus we chose this CASQ as a prototype to further investigate T cell responses to this fusion. While many variants of the TMPRSS2:ERG fusion have been reported, the majority generate frameshifts or initiate transcription downstream from the fusion breakpoint. The TMPRSS2:ERG fusion joining exons 2 and 4 (type VI) is the only predicted variant encoding a CASQ that retains the native reading frame of ERG (Fig. 3B). Furthermore, TMPRSS2:ERG type VI is relatively common amongst the fusion variants, occurring in roughly 10-20% of cases where ERG alterations are present^{3,4}, and is associated with aggressive disease⁵.

Immunogenicity predicted in the TMPRSS2:ERG type VI fusion

The TMPRSS2:ERG type VI fusion was found in 6% of patients (Fig. 4A). *In silico* epitope predictions revealed fusion epitopes predicted to strongly bind the HLA-A*02:01 allele (<50 nM; Fig. 4B). We validated the HLA-A*02:01 binding capacity of TMPRSS2:ERG minimal peptides *in vitro* by an MHC stabilization assay using TAP-deficient T2 cells. Three of the TMPRSS2:ERG minimal peptides stabilized MHC above threshold in this assay, MALNSEALSV, ALNSEALSVV, and ALNSEALSV (Fig. 4C). This coincided with *in silico* epitope predictions, as these were the only TMPRSS2:ERG fusion peptides predicted to bind HLA-A*02:01 with an IC₅₀ <1000nM.

T cells recognize peptides against the TMPRSS2:ERG type VI fusion

We next used TMPRSS2:ERG minimal peptides to stimulate and expand fusion-specific peripheral blood T cells from an HLA-A2 healthy donor. After two rounds of *in vitro* stimulation with peptide-pulsed antigen presenting cells, T cell cultures were assessed for antigen reactivity by IFN γ ELISPOT. We identified three T cell clones that recognized the TMPRSS2:ERG fusion peptides with affinity for HLA-A*02:01 (Fig. 5A). Each clone recognized the 10mer peptide MALNSEALSV, as well as the 9mer contained within (ALNSEALSV). In addition, one clone also recognized the 10mer ALNSEALSVV, suggesting distinct TCR usage from the others two clones. All three T cell lines were primarily CD8⁺ and had upregulated 4-1BB expression in response to peptide stimulation (not shown). T cell lines that were enriched for antigen-specificity were not responsive to the corresponding native TMPRSS2 or ERG peptides (Fig. 5C).

DISCUSSION

Despite the some initial clinical success in melanoma using T cell-based immunotherapy to target the mutanome, the widespread applicability of this approach remains unknown. Moreover, this strategy has focused primarily on somatic point mutations. Whether other mutational events could serve as alternative targets has not been fully examined. In prostate cancer, next-generate sequencing has revealed a paucity of recurrent somatic point mutations though it appears that genetic rearrangements including gene fusions and alternative splicing represents a larger fraction of the overall mutational burden. We undertook a comprehensive *in silico* analysis to identify gene rearrangements that give rise to CASQ, a chimeric amino acid sequence found at the junction of the two native protein sequences. Our systematic approach was conducted using both available computational tools and newly generated algorithms to create a pipeline for high-throughput analysis of CASQ.

Our study revealed novel patient specific and shared CASQ, however, these CASQ were found to generate a low frequency of high-affinity predicted MHC class I epitopes. Several reasons may explain this finding, none of which are mutually exclusive. First, there is a general consensus that prostate tumors are immunologically inert, that is, tumors are classified as suppressive. This stems from several studies showing the presence of T regulatory cells and a low frequency of CD4⁺ or CD8⁺ T cells. In addition, recent evidence has found that hormone-refractory tumors may express a higher frequency of suppressive inhibitor molecules such as PD-L1. The lack of tumor infiltrating effector T cells could point towards tumors that have undergone immune-editing, a possibility that is borne from the slow progression of prostate cancer. Second, there is a new study that suggests androgen therapy can suppress active T cell responses. Whether this is a contributing factor to the lack of predicted CASQ remains unknown. Third, algorithms such as IEDB, while useful, are predictive at best and require vigorous empirical testing to rule out immunogenicity of a given CASQ. Finally, current immunotherapy trials for prostate cancer using a variety of approaches, including dendritic cell-based vaccines (e.g. Provenge), pox-based vaccines (e.g. PROSTVAC) as well as ongoing checkpoint inhibitor trials, have had modest success. Therefore, targeting the mutanome, particularly CASQ will likely require a combinatorial approach involving generation of de novo CASQ targetable by T cells.

This pilot study was conducted in two Phases: (1) Pipeline development and proof of principle analysis, and (2) prospective identification in newly collected tumor specimens. Here, we report the successful completion of Phase 1 and partial completion of Phase 2. As described in the Results section, in Phase 1 we developed and validated a computational pipeline, identified CASQ and predicted HLA epitopes from available databases, and identified T cells that recognize a prototypic CASQ, TMPRSS2:ERG. In Phase 2, we have collected specimens from 3 prospective patients undergoing radical prostatectomy with 2 additional patients awaiting confirmation of their surgical decision. Procured specimens have been cryopreserved or *ex vivo* expanded as per our original proposal. Tumor RNA and TIL TCR Sequencing of the first three patient samples is pending. Finally, we have now gone beyond examining fusions to include alternative spliced genes. We have preliminary data showing that peptides spanning the CASQ of two major spliced isoforms of the androgen receptor that are involved in androgen resistance can be recognized by T cells. This project has

been approved for a one-time extension without additional funds so that we may continue to work towards completion of Phase 2.

KEY ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

1. Training

- trained one 0.5FTE MSc student
- trained one 1.0FTE research assistant

2. Scientific

- first integrated analysis of immunogenic targets comprising chimeric amino acid sequences (CASQ) in PCa.
- first identification of T cells that are reactive to peptides against two CASQ, TMPRSS2:ERG and androgen receptor splice variants.
- in principle demonstration that CASQ are potential antigen targets in prostate cancer.
- study reveals that the low frequency of high-confidence predicted CASQ epitopes in prostate cancer may limit clinical applicability.

3. Manuscripts, abstracts and publications

- 4 abstracts for poster presentation (see Appendix): a) 5th Annual TFRI BC Node Research Day, b) Keystone Symposia – Cancer Immunotherapy: Immunity and Immunosuppression Meet Targeted Therapies, c) Canadian Cancer Immunotherapy Consortium (CCIC) and, d) 2015 ImmunoBC
- 3 oral presentations: 1) Island Prostate Centre (Victoria, BC), 2) Prostate Support Group (Comox/Campbell River, BC) and 3) Fred Hutch Cancer Centre (Seattle, BC).
- *“Chimeric amino acid sequences encoded by gene rearrangements as neoantigens for prostate cancer immunotherapy are low in frequency.”*
Jennifer Kalina, Yen-Yi Lin, David Neilson, Emma Loy, Colin Collins, Davide Salina, Mildred Martens, Paul Blood, Abraham Alexander, Faraz Hach, Julian J. Lum (in preparation)

4. Licenses: n/a

5. Degrees obtained: MSc student 0.5 FTE (MSc defence is pending confirmation in July).

6. Development of resources

- established a new platform at the BC Cancer Agency for obtaining matched fresh radical prostatectomy specimens and peripheral blood for immunological studies

7. Informatics: n/a

8. Funding applied: n/a

9. Employment opportunities: Research Assistant 1.0FTE worked on this project.

10. Appendices

- Abstracts

11. Supporting data

- Figures

CONCLUSIONS

Our study has revealed a high-throughput strategy to identify novel CASQ mutations. While this study has primarily focused on gene fusions, other CASQ targets including alternatively spliced tumor specific variants may be of interest. Despite our successfully implementing this computational pipeline, the low mutational events related to CASQ in prostate cancer may limit T cell-based approach to target these neoantigens.

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Abstract: CHIMERIC PROTEINS AS IMMUNE TARGETS IN PROSTATE CANCER

Jennifer L. Kalina^{1,2}, David S. Neilson^{1,2}, Julie S. Nielsen¹, Spencer D. Martin^{1,3,4}, Julian J. Lum^{1,2}

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Background: Cancer vaccines aim to elicit antigen-specific T cell responses against tumor antigens. Most prostate cancer vaccines to date target mis-expressed or over-expressed proteins; however, these proteins are often dispensable for the tumor, allowing for antigen escape, or have tolerance mechanisms in place that may curb induction of T cell immunity. Recent studies provide compelling evidence that tumor-specific mutations are a novel source of T cell targetable antigens (neoantigens). Metastatic Castration Resistant Prostate Cancers (mCRPC) contain several recurrently mutated fusion proteins that may serve as viable immune targets. The TMPRSS2:ERG fusion protein is found in a large proportion of mCRPC, is involved in several oncogenic pathways, and predicts poor overall survival; thus, this fusion is likely functionally important for tumor maintenance, progression, and metastasis.

Hypothesis: Gene fusions, such as TMPRSS2:ERG, generate chimeric amino acid sequences that are targetable by T cells.

Methods and Results: With this aim, we pulsed autologous dendritic cells with peptides corresponding to the TMPSS2:ERG type VI fusion site to activate and expand naïve fusion-specific T cells from peripheral blood of healthy donors. After two rounds of stimulation, expanded T cell cultures were assessed by interferon- γ ELISPOT for recognition of fusion peptides. T cell responses to two epitopes spanning the TMPRSS2:ERG fusion were confirmed in an HLA-A*02:01 healthy donor. These two peptides were predicted to bind HLA-A*02:01, which was confirmed by MHC stabilization assays. Currently, we are assessing whether these minimal peptides are naturally processed as well as whether antigen-specific T cell clones can lyse tumor cells that express the TMPRSS2:ERG type VI fusion protein.

Conclusions and Future Directions: Future studies will assess TMPRSS2:ERG positive mCRPC patients for the presence of pre-existing T cell responses to this fusion. Our findings to date have implications for the use of fusions as T cell targetable epitopes for therapeutic vaccination against fusion oncogenes in prostate cancer.

IMMUNOGENICITY OF CHIMERIC AMINO ACID SEQUENCES IN PROSTATE CANCER

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Cancer vaccines aim to generate robust antigen-specific T cell responses against tumor antigens. Currently, most available prostate cancer vaccines target over-expressed native proteins that are often not essential for tumor maintenance or progression, thus providing avenues for antigen escape. Furthermore, these targets resemble self antigens and may be naturally tolerated by the immune system. Recent studies show that tumor mutations are a novel source of T cell antigens. In prostate cancer, chromosomal translocations and gene fusions are common and generate novel chimeric amino acid sequences (CASQ) that may serve as ideal immune targets. One such mutation is the TMPRSS2:ERG fusion, found in approximately 50% of prostate cancer cases. TMPRSS2:ERG is involved in several oncogenic pathways and predicts poor overall survival; thus, this fusion may be functionally important for tumor progression and metastasis. We used monocyte-derived dendritic cells to present TMPRSS2:ERG type VI fusion peptides to T cells from the peripheral blood of healthy donors. After two rounds of stimulation, T cell cultures were assessed for recognition of fusion peptides by IFN- γ ELISPOT. T cell responses to two epitopes spanning the fusion were confirmed in an HLA-A2 healthy donor. These two peptides were predicted and confirmed binders of HLA-A*02:01. Currently, we are assessing whether TMPRSS2:ERG minimal peptides are endogenously processed and presented on MHC-I and will also determine whether TMPRSS2:ERG-specific T cells recognize TMPRSS2:ERG-expressing tumor cells. Our findings have implications for the use of CASQ as T cell targetable epitopes in immunotherapeutic strategies against prostate cancer.

ImmunoBC 2015 Abstract form

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<p>IMMUNE TARGETING OF CHIMERIC PROTEINS IN PROSTATE CANCER</p> <p><u>Jennifer L. Kalina</u>^{1,2}, David S. Neilson^{1,2}, Julie S. Nielsen¹, Spencer D. Martin^{1,3,4}, Julian J. Lum^{1,2}</p> <p>¹Trev and Joyce Deeley Research Centre, BC Cancer Agency, Victoria, BC, Canada, ²Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada, ³Genome Sciences Centre, Vancouver, BC, Canada, ⁴Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada</p> <p>Background: Cancer vaccines aim to elicit antigen-specific T cell responses against tumor antigens. Most prostate cancer vaccines to date target mis-expressed or over-expressed proteins; however, these proteins are often dispensable for the tumor, allowing for antigen escape, or have tolerance mechanisms that may curb induction of T cell immunity. Recent studies provide compelling evidence that tumor-specific mutations are a novel source of T cell antigens, so called neoantigens. Metastatic Castration Resistant Prostate Cancers (mCRPC) contain several recurrently mutated fusion proteins that may serve as viable immune targets. One such mutation is the TMPRSS2:ERG fusion, which is found in about 50% of mCRPC. TMPRSS2:ERG is involved in several oncogenic pathways and predicts poor overall survival; thus, this fusion may be functionally important for tumor maintenance, progression, and metastasis.</p> <p>Hypothesis: Gene fusions, such as TMPRSS2:ERG, generate chimeric amino acid sequences that are targetable by T cells.</p> <p>Methods and Results: We used monocyte-derived dendritic cells to present overlapping minimal peptides corresponding to the TMPRSS2:ERG type VI fusion site to activate and expand naïve fusion-specific T cells from the peripheral blood of healthy donors. After two rounds of stimulation, expanded T cell cultures were assessed by interferon-γ ELISPOT for recognition of fusion peptides. T cell responses to two epitopes spanning the TMPRSS2:ERG fusion were confirmed in an HLA-A*02 healthy donor. These two peptides were predicted to bind HLA-A*02:01, which was confirmed by MHC stabilization assays. Currently, we are assessing whether these minimal peptides are naturally processed as well as whether antigen-specific T cell clones can lyse tumor cells that express TMPRSS2:ERG.</p> <p>Conclusions and Future Directions: Future studies will assess TMPRSS2:ERG positive mCRPC patients for the presence of pre-existing T cell responses to this fusion. Our findings to date have implications for the use of fusions as T cell targetable epitopes in immunotherapeutic strategies against prostate cancer.</p>	

IMMUNE TARGETING OF CHIMERIC PROTEINS IN PROSTATE CANCER

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Background: Cancer vaccines aim to generate robust antigen-specific T cell responses against tumor antigens. To date, most available prostate cancer vaccines target native proteins that are mis-expressed or over-expressed in the tumor. These target proteins are often not essential for tumor maintenance or progression, thus allowing the possibility for antigen escape. Furthermore, since these targets resemble self antigens, they may be naturally tolerated by the immune system. Recent studies provide compelling evidence that tumor-specific mutations are a novel source of T cell antigens. Metastatic Castration Resistant Prostate Cancers (mCRPC) contain several recurrently mutated genomic fusions, which generate proteins that may serve as viable immune targets. One such mutation is the TMPRSS2:ERG fusion, found in approximately 50% of mCRPC. TMPRSS2:ERG is involved in several oncogenic pathways and predicts poor overall survival; thus, this fusion may be functionally important for tumor maintenance, progression, and metastasis.

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Methods and Results: We used monocyte-derived dendritic cells to present overlapping minimal peptides, corresponding to the TMPSS2:ERG type VI fusion site, to activate and expand naïve fusion-specific T cells from the peripheral blood of healthy donors. After two rounds of stimulation, expanded T cell cultures were assessed by interferon-γ ELISPOT for recognition of fusion peptides. T cell responses to two epitopes spanning the TMPRSS2:ERG fusion were confirmed in an HLA-A*02 healthy donor. These two peptides were predicted to bind HLA-A*02:01, which was confirmed by MHC stabilization assays. Currently, we are assessing whether these minimal peptides are naturally processed and presented on MHC I using *in vitro* transcribed mRNA (IVT-RNA) transfected into autologous B cells. We will also determine whether antigen-specific T cell clones can lyse tumor cells expressing TMPRSS2:ERG.

Conclusions and Future Directions: Future studies will assess TMPRSS2:ERG positive mCRPC patients for the presence of pre-existing T cell responses to this fusion. Our findings to date have implications for the use of fusions as T cell targetable epitopes in immunotherapeutic strategies against prostate cancer.

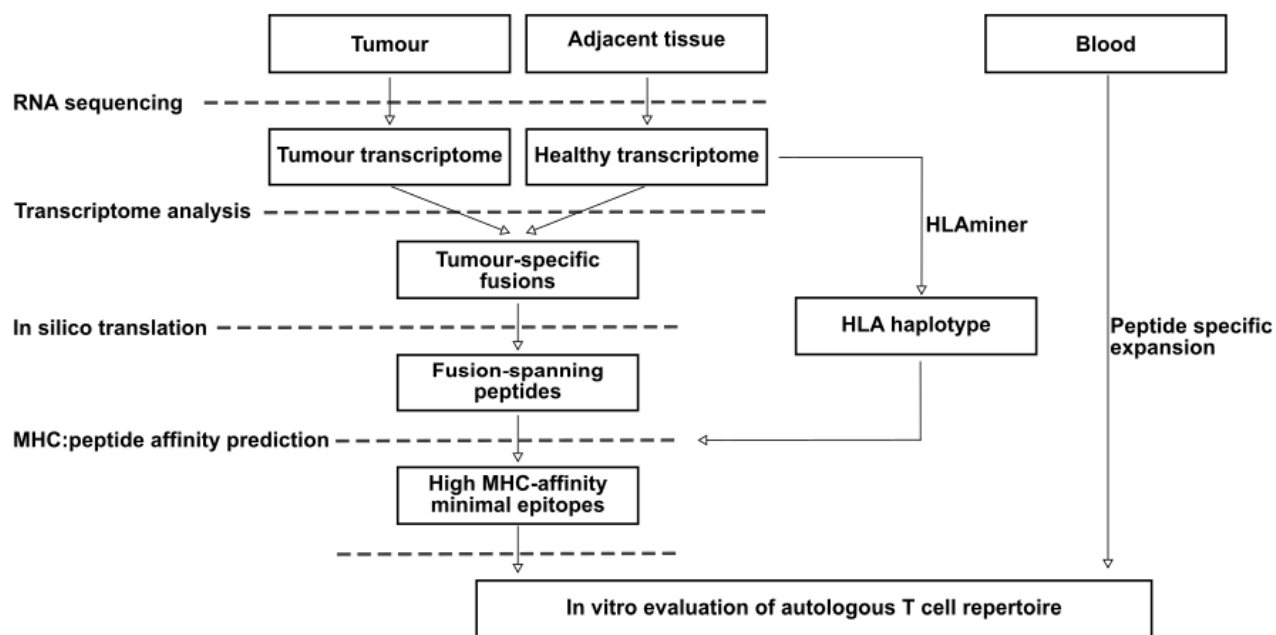
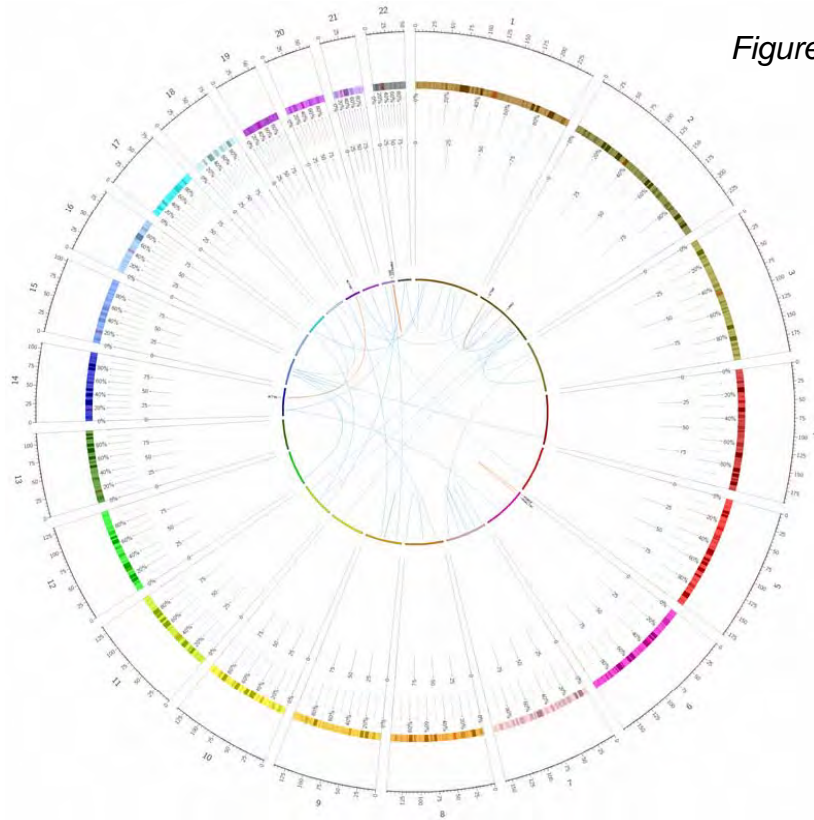
A

Figure 1. Computational pipeline to identify high-confidence immunogenic CASQ. Data is generated from RNA-sequencing from tumor and adjacent normal tissue. Transcriptomic analysis reveals tumor-specific gene fusions and in silico translation to generate high-confidence CASQ. Patient HLA is determined via HLAminer to generate a list of MHC:peptide affinity prediction scores. In matched blood, peptides are evaluated for autologous T cells that recognition the CASQ.

Figure 1. Lum (PC141070)

B



C

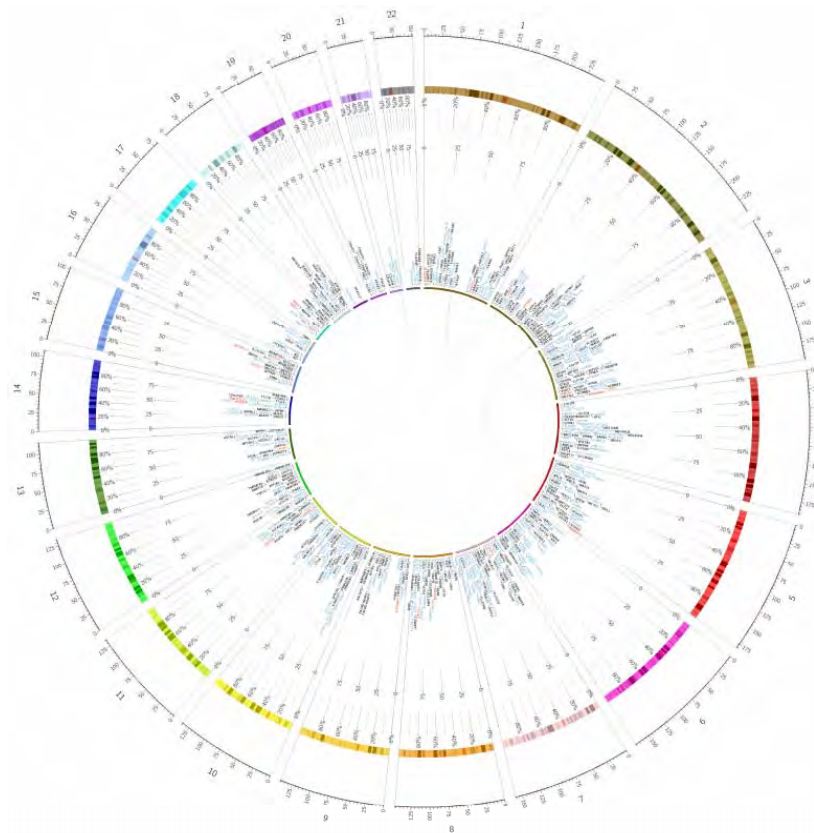


Figure 1 (Con'd). High-confidence gene fusions in tumor LTL-331-7. (B) Circos plot showing the total number gene fusions. **(C)** Circos plot showing gene fusions between exon boundaries

Figure 2. Lum (PC141070)

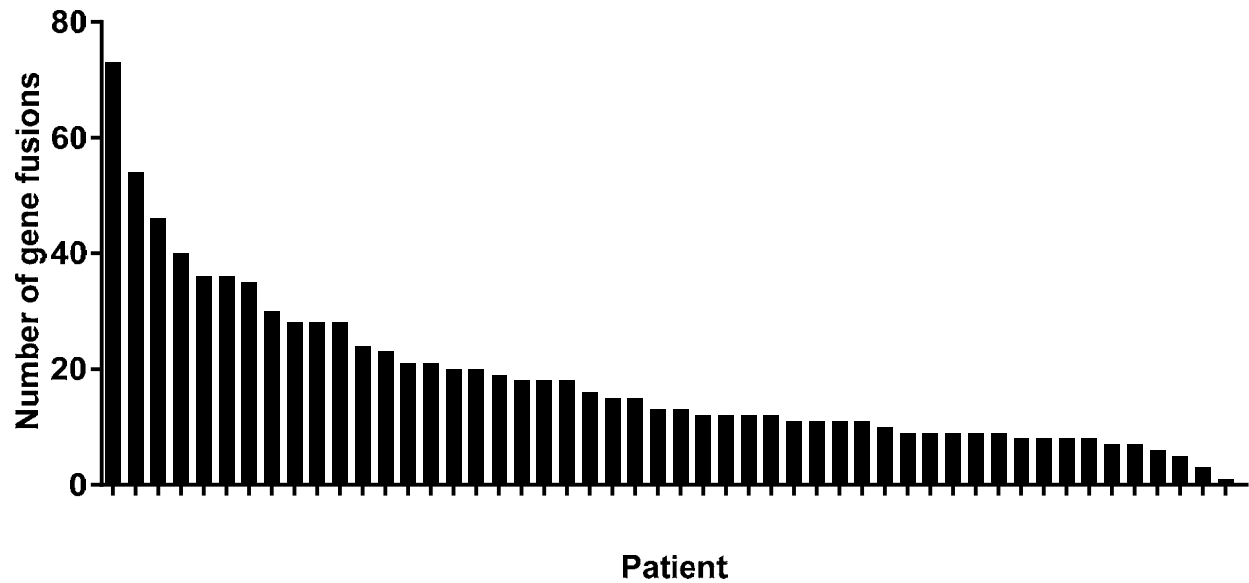
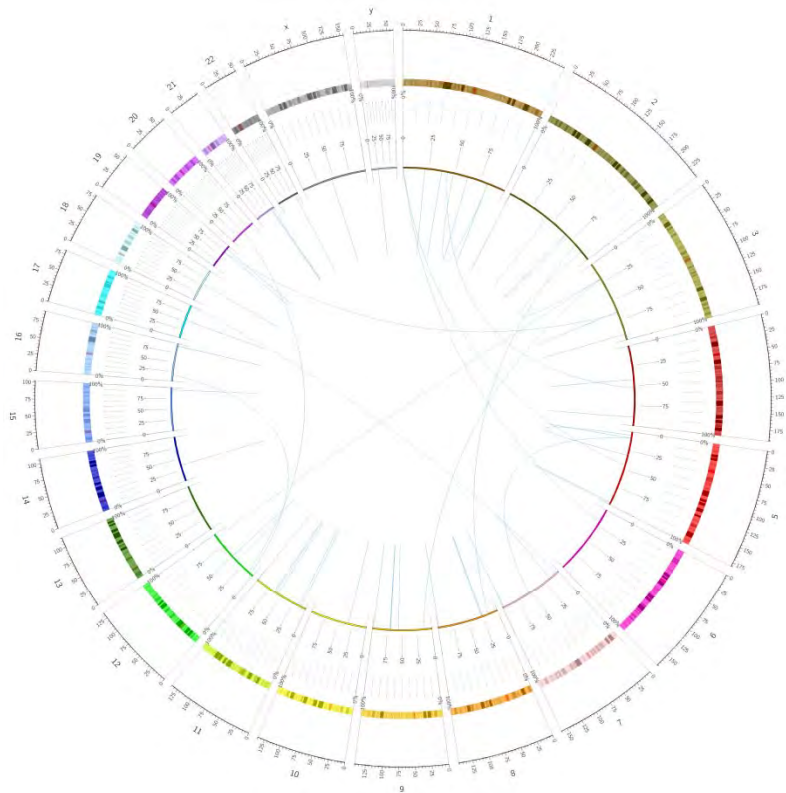


Figure 2. High-confidence gene fusions in tumor LTL-331-7. (A) Circos plot showing the total number gene fusions. **(B)** Circos plot showing gene fusions between exon boundaries

Figure 3. Lum (PC141070)

C



D

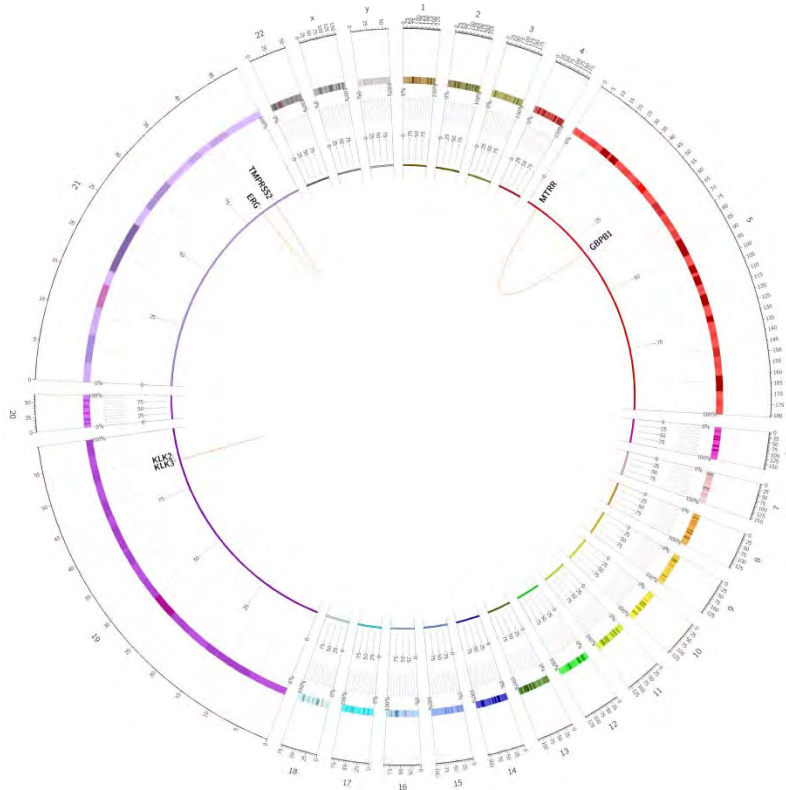
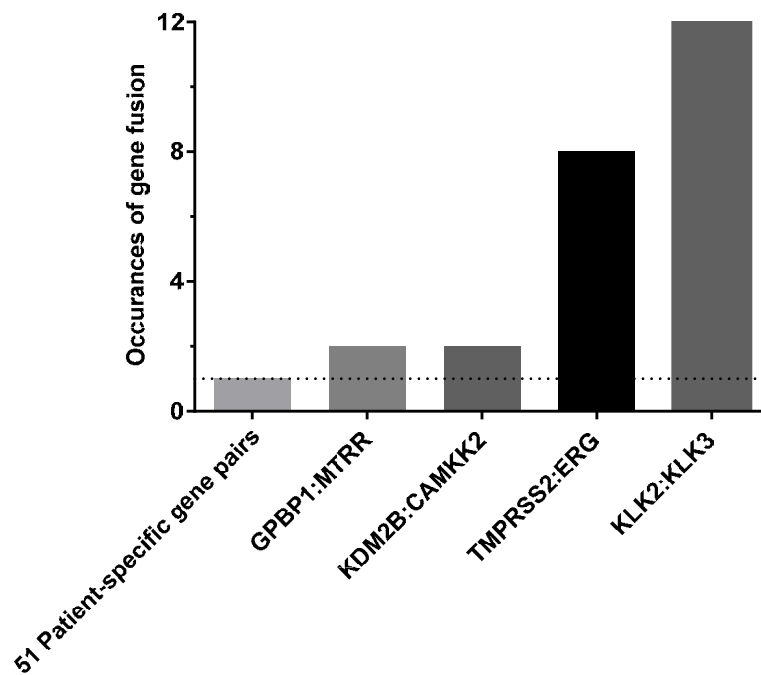


Figure 3. High-confidence gene fusions in tumor TCGA-EJ-7327. (A) Circos plot showing the total number gene fusions. **(B)** Circos plot showing gene fusions between exon boundaries

Figure 3. Lum (PC141070)

A



B

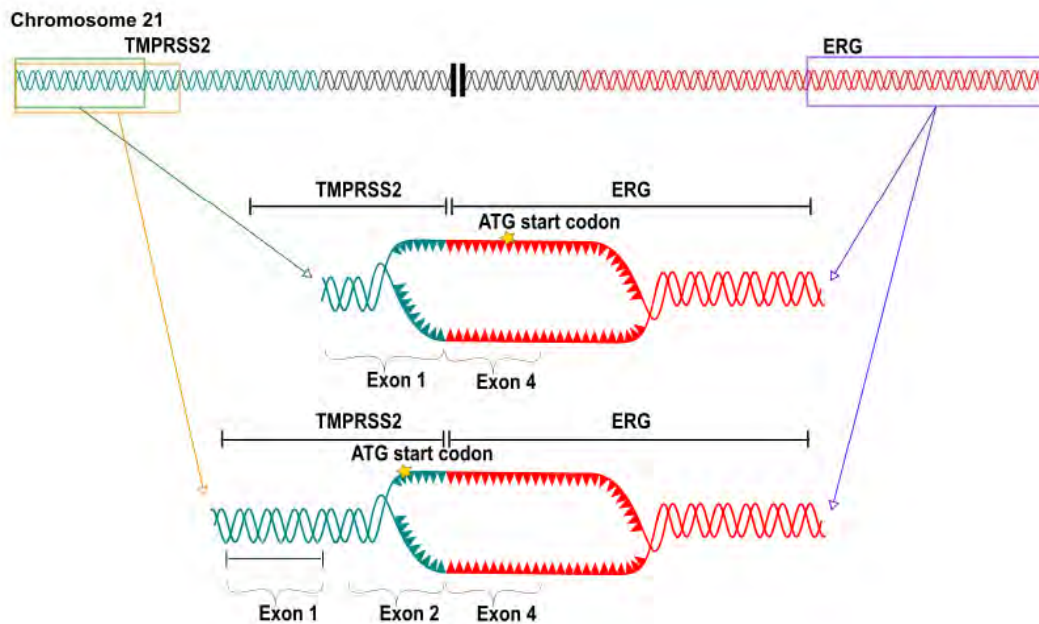


Figure 4. Recurrent fusion genes present in the TCGA intermediate and high risk patient dataset. (A) Occurrence of 4 fusions across patient dataset (n=51). **(B)** TMPRSS:ERG type VI fusion.

Figure 4. Lum (PC141070)

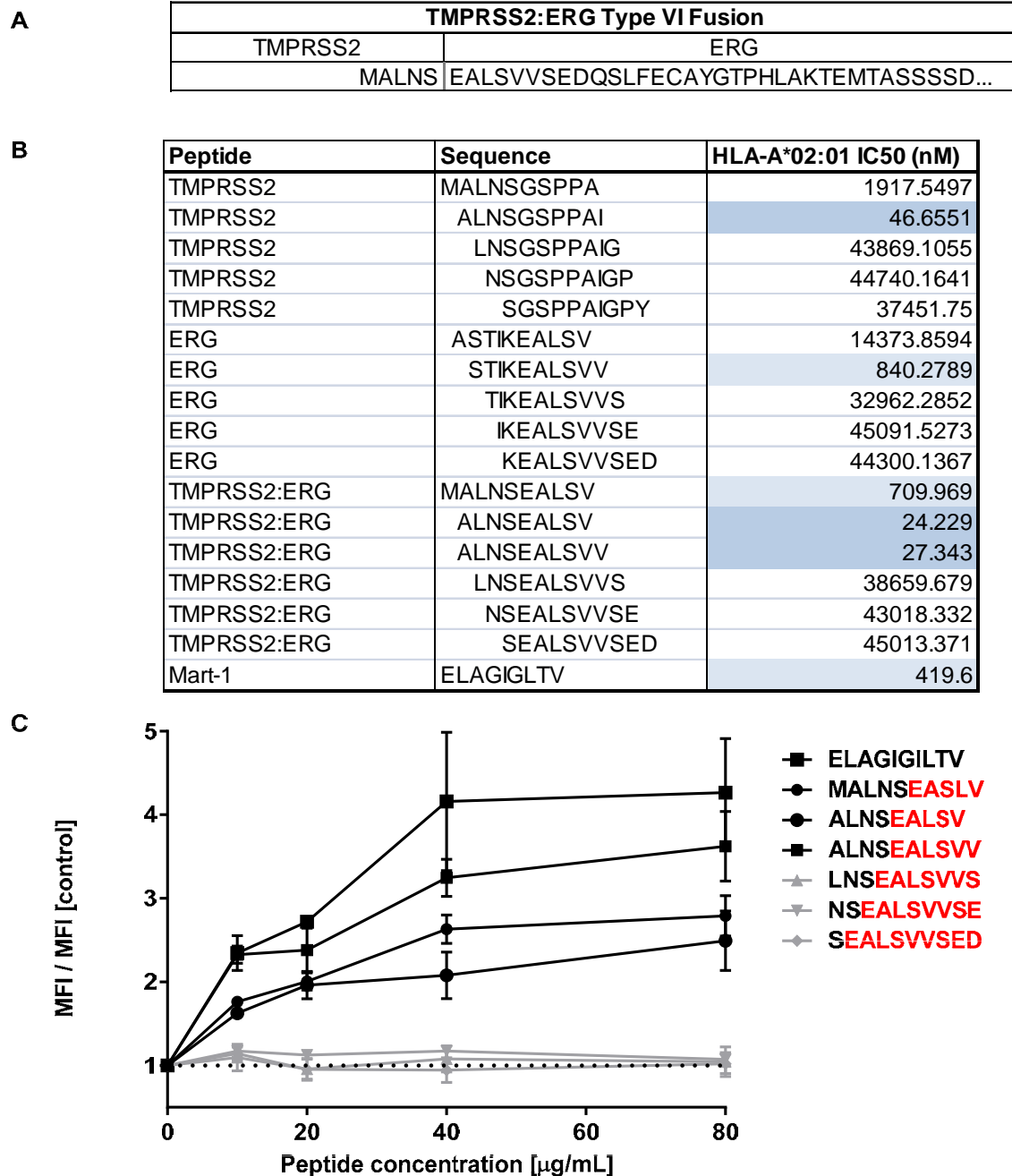


Figure 6. *TMPRSS2:ERG* peptides bind HLA-A*02:01. (A) *TMPRSS2:ERG* amino acid sequence (B) IC50 scores from MHC epitope prediction software for each of the *TMPRSS2:ERG* peptides. Data includes all possible fusion-spanning 10mers, as well as the sole 9mer with a high-affinity binding score. Lower IC50 scores indicate an increased likelihood of peptide binding to HLA-A*02:01. Highlighted scores indicate peptides which meet the affinity threshold for predicted binding to HLA-A*02:01. (C) *TMPRSS2:ERG* peptides MALNSEALSV, ALNSEALSV, and ALNSEALSVV each stabilize HLA-A*02:01. T2 cells were pulsed with increasing concentration of each peptide for 18 hours at 26°C followed by 3 hours at 37°C in the presence of 10μg/mL brefeldin A. Cells were stained with anti-HLA-A*02 FITC for 30 minutes at 4°C and analyzed for MHC stabilization by FACS. The mean fluorescence index relative to unpulsed T2 cells is shown. The HLA-A*02:01-restricted peptide from MART-1, ELAGIGLTV, was used as a positive control.

Figure 5. Lum (PC141070)

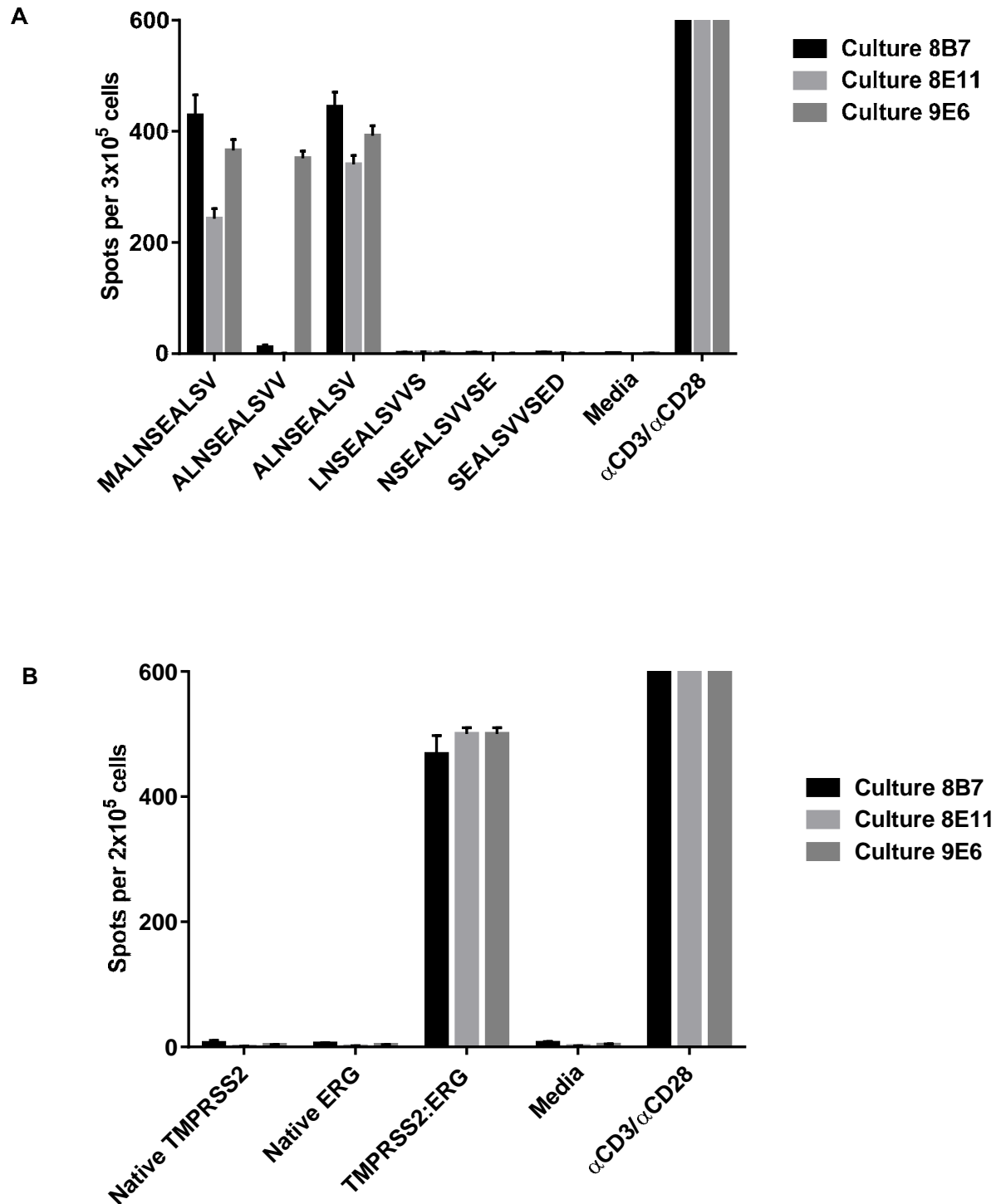


Figure 7. (A) As determined by IFN γ ELISpot, a subset of healthy donor T cells are activated upon incubation alongside TMPRSS2:ERG peptides with high affinity for HLA-A*02:01. (B) As determined by IFN γ ELISpot, TMPRSS2:ERG-Specific T cells do not cross-react with the native TMPRSS2 or ERG peptides. T cells were incubated with pools of overlapping peptides from native TMPRSS2, ERG, and TMPRSS2:ERG at 10 μ g/mL for 20 hours and assessed for IFN γ production. (Note: native peptide sequences correspond to the junction point sequence of TMPRSS2:ERG)